June 9, 2015

The HRP cDNA was received, and suspended by following the provided protocol

- Centrifuging the tube for 3-5 seconds at a minimum speed of 3000 x g
- Adding 100µL of TE to reach a final concentration of 10ng/µL
- Incubating the gene at 50˚C for 20 min.
- Vortex and centrifuge

* the same protocol was followed for suspending the primers but the concentration was different in that it was measured in µM. the concentration of the primer prefix (26.8 nmoles), and suffix (36.3 nmoles), was 100µM, which was diluted to 10µM by adding 268 µL and 363 µL of TE to the primers, respectively.

The next step was to perform a PCR to amplify the gene sequence along with the Biobrick prefix and suffix.

Data:

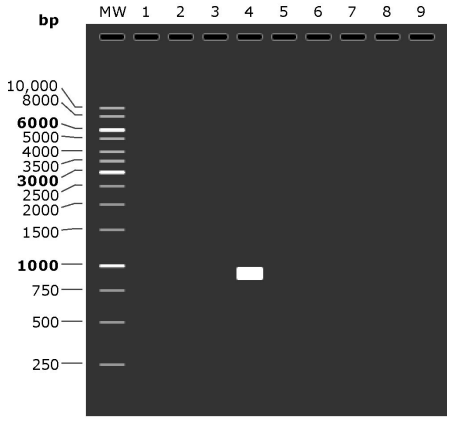
HRP PCR—

Materials	Volume (µL)
Master Mix	25
F primer	1
R Primer	1
HRP DNA	1
H2O	25.5
Total	53.5

June 10, 2015

Data:

Predicted -



Gel results of HRP PCR seemed to show correct band size. Should have ran another to double check but forgot.

Did PCR purification using Thermo Scientific gel extract and cleanup kit. Followed protocol for enzymatic cleanup instead of primer dimer removal as there didn't seem to be any primer dimers on gel.

A. General DNA cleanup from enzymatic reactions protocol.

1 Adjust the volume of the reaction mixture to 200 µL with Water, nuclease-free or TE buffer (not included).

2 Add 100 µL of Binding Buffer. Mix thoroughly by pipetting.

3 Add 300 µL of ethanol (96-100%) and mix by pipetting.

4 Transfer the mixture to the DNA Purification Micro Column preassembled with a collection tube. Centrifuge the column for 30-60 seconds at 14,000 × g. Discard the flow-through. Place the DNA Purification Micro Column back into the collection tube. Note. 1. If DNA fragment is ≥ 10 kb centrifuge the column for 2 minutes at 14,000 × g. 2. Close the bag with DNA Purification Micro Columns tightly after each use!

5 Add 700 µL of Wash Buffer (supplemented with ethanol, see p. 3) to the DNA Purification Micro Column and centrifuge for 30-60 seconds at 14,000 × g. Discard the flow-through and place the purification column back into the collection tube. Note. If DNA fragment is ≥ 10 kb centrifuge the column for 2 minutes at 14,000 × g.

6 Repeat step 5.

7 Centrifuge the empty DNA Purification Micro Column for an additional 1 minute at 14,000 × g to completely remove residual Wash Buffer. Note. This step is essential to avoid residual ethanol in the purified DNA solution. The presence of ethanol in the DNA sample may inhibit downstream enzymatic reactions.

8 Transfer the DNA Purification Micro Column into a clean 1.5 mL microcentrifuge tube (not included).

9 Add 10 µL of Elution Buffer to the center of the DNA Purification Micro Column membrane. Centrifuge for 1 minute at 14,000 × g to elute DNA. Note. • If DNA fragment is ≥ 10 kb the elution volume should be increased to 15-20 µL to get optimal DNA yield. • Lower volume of Elution Buffer for DNA Micro Kit can be used (6-10 µL) in order to concentrate eluted DNA. Please notice that < 10 µL elution volume slightly decreases DNA yield. • Double the elution volume or perform two elution cycles when purifying larger amounts of DNA (for example > 5 µg). **Added 20ul EB instead of 10..spec'd..then added 15 more. For now on just go with 25ul EB when using this protocol**

10 Discard the purification column and store the purified DNA at -20°C

Results –

First 20ul – 462 ng/ul concentration

- A260 – 0.185
- A280 – 0.101
- 1.832 ratio

Next 15ul – 150ng/ul

- A260 – 0.055
- A280 – 0.028
- 1.964

20ul and 15ul are in different eppendorfs in freezer

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pSB1C3 Linear Digest

Made enzyme master mix for plasmid backbone by following protocol on iGEM website. 25ul total. No DpnI.

Digest

- Enzyme Master Mix for Plasmid Backbone (25ul total, for 6 rxns)
  - 5 ul NEB Buffer 2
  - 0.5 ul BSA
  - 0.5 ul EcoRI-HF
  - 0.5 ul PstI
  - 0.5 ul DpnI (Used to digest any template DNA from production)
  - 18 ul dH20
- Digest Plasmid Backbone
  - Add 4 ul linearized plasmid backbone (25ng/ul for 100ng total)
  - Add 4 ul of Enzyme Master Mix
  - Digest 37C/30 min, heat kill 80C/20 min

Then ran two digestions of linearized plasmid using above protocol. Combined after heat kill to make 16 ul of 12.5ng/ul digested plasmid

---

Constructs

Problems with HRP and CBDA Synthase constructs and biobrick prefix and suffix primers. These constructs only consist of cDNA plus biobrick assembly standard 10 prefix and suffix. Having the Eco and Pst cut sites on the ends of the DNA inhibit digestions. Enzymes need a few nucleotides to bind to.

Also the biobrick primers are binding at both prefix and suffix because they are palindromes. Biobrick prefix forward primer can bind to suffix and go in reverse. Biobrick suffix reverse primer can bind to prefix and go forward. This is resulting in multiple (4) different PCR products. Only one of which with proper prefix and suffix restriction sites.

Need to make new constructs and primers.

June 11, 2015

Digests -

Today we digested some of the purified HPR PCR product with 462ng/ul concentration plus some of the HPR from 10ng/ul stock.

Digest 1- HRP stock (20ul)

- 10 ul HRP stock
- 3ul Cutsmart Buffer
- 0.5 ul EcoRI-HF
- 0.5 ul PstI-HF
- 6 ul dH2O

Digest 2- PCR product (50ul)

- 4 ul HRP PCR
- 5 ul Cutsmart Buffer
- 1 ul EcoRI-HF
- 1 ul PstI-HF
- 39 ul H2O

Both digests ran at 37 C for 2 hours and then heat killed at 80 C for 20 mins.

Digested stock - 42.5 ng/ul , 0.944 OD260/280

Digested PCR - 90.0 ng/ul , 1.286 OD260/280

Made Chloramphenicol plates.

1. Add the following to 800ml H_2O
    - 10g Bacto-tryptone
    - 5g yeast extract
    - 10g NaCl
2. Adjust pH to 7.5 with NaOH
3. Add 15g agar
4. Melt agar into solution on hot plate.
5. Adjust volume to 1L with dH_2O
6. Sterilize by autoclaving.
7. Once cooled to touch, add 1ml chloro, mix, pour 20-25 mL of broth into each plate.

Constructs

Created new HRP and CBDA synthase constructs + primers. Works in snapgene but needs to be checked by Jess.

Added additional nucleotides on both ends of the old constructs. Enables better binding by restriction enzymes and specificity for the primers. Made new primers - IDT Primer F and IDT Primer R. Primer F binds to prefix plus additional nucleotides, while Primer R binds to suffix plus nucleotides. We can add same nucleotides to all constructs from IDT and use same primers. Standardized. Also only 1 PCR product expected instead of 4.

June 15, 2015

Goal: to ligate HRP (source & PCR product) into iGEM linearized pSB1C3. Also to perform transformation of the HRP plasmid into E.coli (SIG10-alpha), and plate the samples.

Ligations

HRP into pSB1C3. 2 ligations, 1 with source HRP and 1 from PCR'd HRP.

10 ul ligation volumes

- 2ul digested pSB1C3
- 1ul digested HRP
- 0.5ul T4 DNA Ligase
- 1ul 10x Ligase Buffer
- 5.5ul nuclease free H2O

Ligate 22 C / 15 mins, heat kill 65 C / 10 mins

Took 6 ul from each ligation and did 4 transformations at 3 ul per. Then added 0.3 ul T4 Ligase to remaining 4 ul and ran overnight ligation at 16 C / 16 hours + heat kill 65 C / 15 mins.

Transformations

Used SIG10-alpha chemically competent cells with included recovery medium. Protocol found on company site. Plates left overnight.

To ensure successful transformation results, the following precautions must be taken:

- **For best results, ligation reactions must be heat killed at 70ºC for 15 minutes before transformation. Alternately, the reactions may be purified, if desired.**
- **Prepare nutrient agar plus antibiotic.**
- **All microcentrifuge tubes must be thoroughly pre-chilled on ice before use.**
- **The cells must be completely thawed on ice before use.**
- **For highest transformation efficiency, use the provided Recovery Medium to resuspend the cells after transformation.**

Transformation Protocol

1. **Prepare nutrient agar plates with appropriate antibiotic.**
2. **Chill sterile culture tubes on ice (17 mm x 100 mm tubes, one tube for each transformation reaction).**
3. **Remove SIG10 5-alpha cells from the -80°C freezer and thaw completely on wet ice (10-20 minutes).**
4. **Add 40 µl of the cells to the chilled culture tube.**
5. **Add 1-4 µl of ligation reaction or DNA sample to the 40 µl of cells on ice. (Failure to purify or heat-inactivate, or otherwise purify, the ligation reaction may prevent transformation.) Stir briefly with pipet tip; do not pipet up and down to mix, which can introduce air bubbles and warm the cells.**
6. **Incubate on ice for 30 minutes.**
7. **Heat shock cells by placing them in a 42oC water bath for 30 seconds.**
8. **Return the cells to ice for 2 minutes.**
9. **Add 950 µl of room temperature Recovery Medium to the cells in the culture tube.**
10. **Place the tubes in a shaking incubator at 250 rpm for 1 hour at 37 oC.**
11. **Plate up to 100 µl of transformed cells on nutrient agar plates containing the appropriate antibiotic.**
12. **Incubate the plates overnight at 37°C.**
13. **Transformed clones can be further grown in any rich culture medium.**

- See more at:
http://www.sigmaaldrich.com/technical-documents/protocols/biology/sig10-5-alpha-chemically-competent-cells.html#sthash.zNKwF20I.dpuf

June 16, 2015

Goal: to perform colony PCRs on the plated samples (HRP in pSB1C3), run the samples on a gel for confirmation. Transform overnight ligations. Confirm overnight ligations with PCR + gel.

HRP + pSB1C3 plates showed no growth. No colony PCR ran. Transformed overnight ligations into E. coli (SIG10-alpha with same protocol) and plated. Also ran confirmation PCR + gel on ligated DNA.

PCR + Gel of Overnight Ligations

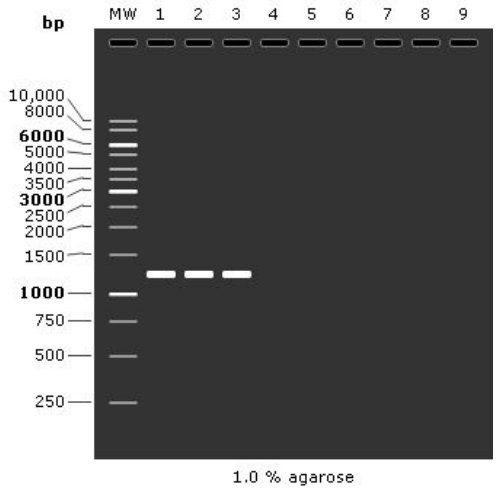
2x. Took 1 ul from each ligation reaction (HRP source and HRP PCR). 30 ul final volumes.

- 1ul HRP in pSB1C3
- 1ul Vf Primer
- 1ul Vr Primer
- 12.5ul Master Mix
- 14.5ul dH2O

Standard Vf/Vr program in thermocycler. 94 C -> 50 C -> 72 C. 35 cycles.

Split PCR products into 15ul/15ul/7ul samples. Unfortunately all but 7ul of HRP source in pSB1C3 evaporated during PCR due to PCR tube opening. Added 3ul/3ul/2ul Loading Dye. Loaded into 1% agarose gel and ran at 80V for 80 mins.

Predicted result-



Actual result - 1 strong band at around 300 bps for the 7ul source HRP + pSB1C3. Light bands at around 300 bps for the 15ul PCR HRP + pSB1C3s. Overnight Ligations don't show expected product.

Furthermore, a second attempt was made to transform the HRP pSB1C3 vector into E.coli (SIG10-alpha with the same protocol). Also, a positive control of RFP pSB1C3 was transformed into E.coli as well. The transformed cells were incubated for an overnight growth to be further analyzed through colony PCR.

June 17, 2015

Plates showed no growth after 48 hours incubation time. Overnight ligations showed no growth after 24 hours incubation. Ordered new HRP construct with added nucleotides to improve digestions and enable better PCR results. Also ordered new standardized primers for all IDT parts.

Constructs

Ordered CBDA synthase, HRP, Mambalgin, and alpha secretion constructs. All in assembly standard 10 with standardized additional nucleotides. Also ordered IDT Primer F and IDT Primer R.

June 18, 2015

The plates for the second attempted transformation of HRP + pSB1C3 and RFP (positive control) showed no growth after 48 hours of incubation. All plates thrown out.

Constructs

Canceled mambalgin and alpha secretion constructs after further thought. Mambalgin had wrong prefix and suffix while alpha secretion did not feel right. Need to research other assembly standards.

June 22, 2015

Constructs

Made and ordered mambalgin and alpha secretion constructs and decided to use assembly standard 25 (Frieburg Standard) instead of the normal assembly standard 10. Biobrick prefix and stuff have additional restriction sites (AgeI and NgoMIV) which enable the creation of fusion proteins. Cutting at AgeI and NgoMIV and ligating creates a stop-less scar (no ATG)..unlike cutting at Xba and Spe which created a scar with a stop codon.

IDT primers will still work with new constructs as standard additional nucleotides were also added.

All 4 new constructs should be arriving at around June 29th or 30th.

Old HRP/CBDA synthase constructs PCR'd with IDT Primers

IDT Primers might add additional nucleotides to old constructs. Did 2 reactions (1 each of HRP and CBDA synthase)

- 25ul Master Mix
- 1ul IDT Primer F
- 1ul IDT Primer R
- 1ul HRP/CBDA synthase
- 22ul dH2O

Melting temp only at 43 C. Ran 95 C -> 43 C -> 73 C for 35x cycles.

Put resulting PCR'd mix in 1% agarose gel at 80V for 80 mins.

Results showed strong band for HRP at right length and very faint band for CBDA synthase at correct length. Realized 43 C melting temp was only for first primer annealing..then will increase to 53 C once additional nucleotides added. Will try again.

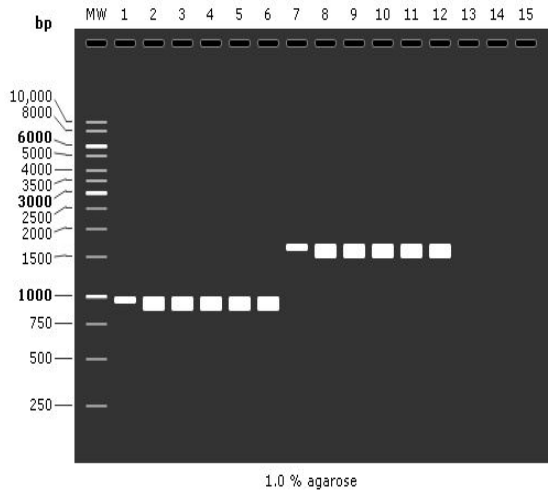
June 24, 2015

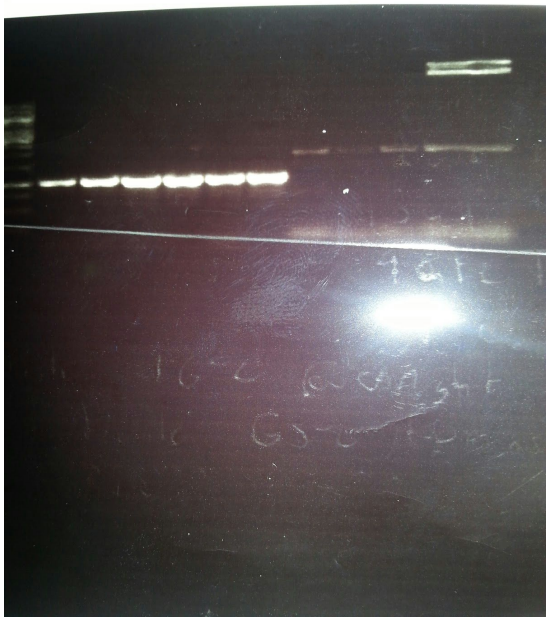
PCR

Mudassar did 12 PCR reactions (6 of HRP and 6 of CBDA synthase) at 50ul final volume. Plan is to gel extract, digest, and use for ligations.

- 25ul Master Mix
- 1ul IDT Primer F
- 1ul IDT Primer R
- 1ul HRP/CBDA synthase
- 22ul dH2O

This time did 5 cycles at 43 C then 35 cycles at 53 C.





Results again showed strong bands for HRP at correct length, while CBDA synthase bands were very faint. Could be because CBDA synthase is ~1700 bps while HRP is ~1000. Regular master mix/taq polymerase not good for longer strands. Will try high fidelity version master mix on cbda synthase later.

Gel Extraction

Followed the DNA extraction from gel protocol from the Thermo Scientific Gel Extraction and DNA Cleanup Micro Kit.

1 Excise up to 200 mg gel slice containing the DNA fragment using a clean scalpel or razor blade. Cut as close to the DNA as possible to minimize the gel volume. Place the gel slice into a 1.5 mL tube.

Note. If the purified fragment will be used for cloning reactions, avoid damaging the DNA through UV light exposure. Minimize UV exposure to a few seconds or keep the gel slice on a glass or plastic plate during UV illumination.

2 Add 200 µL of Extraction Buffer. Mix thoroughly by pippeting.

3 Incubate the gel mixture at 50-58°C for 10 minutes or until the gel slice is completely dissolved. Mix the tube by inversion every few minutes to facilitate the melting process. Ensure that the gel is completely dissolved. Note. For > 1% agarose gels prolong the incubation time to 15 min.

4 Add 200 µL of ethanol (96-100%) and mix by pipetting.

5 Transfer the mixture to the DNA Purification Micro Column preassembled with a collection tube. Centrifuge the column for 30-60 seconds at 14,000 × g. Discard the flow-through. Place the DNA Purification Micro Column back into the collection tube. Note. 1. If DNA fragment is ≥ 10 kb centrifuge the column for 2 minutes at 14,000 × g. 2. Close the bag with DNA Purification Micro Columns tightly after each use!

6 Add 200 µL of Prewash Buffer (supplemented with ethanol, see p. 3) to the DNA Purification Micro Column and centrifuge for 30-60 seconds at 14,000 × g. Discard the flow-through and place the purification column back into the collection tube. Note. If DNA fragment is ≥ 10 kb centrifuge the column for 1-2 minutes at 14,000 × g.

7 Add 700 µL of Wash Buffer (supplemented with ethanol, see p. 3) to the DNA Purification Micro Column and centrifuge for 30-60 seconds at 14,000 × g. Discard the flow-through and place the purification column back into the collection tube. Note. If DNA fragment is ≥ 10 kb centrifuge the column for 1-2 minutes at 14,000 × g.

8 Repeat step 7.

9 Centrifuge the empty DNA Purification Micro Column for an additional 1 minute at 14,000 × g to completely remove residual Wash Buffer. Note. This step is essential to avoid residual ethanol in the purified DNA solution. The presence of ethanol in the DNA sample may inhibit downstream enzymatic reactions.

10 Transfer the DNA Purification Micro Column into a clean 1.5 mL microcentrifuge tube (not included).

11 Add 10 µL of Elution Buffer (**Added 20ul**) to the DNA Purification Micro Column. Centrifuge for 1 minute at 14,000 × g to elute DNA. Note. • If DNA fragment is ≥ 10 kb the elution volume should be increased between 15-20 µL to get optimal DNA yield. Elution volume less than 10 µL is not recommended. • Lower volume of Elution Buffer for DNA Micro Kit can be used (6-10 µL) in order to concentrate eluted DNA. Please notice that < 10 µL elution volume slightly decreases DNA yield. 12 Discard the purification column and store the purified DNA at -20°C.

Results

All 6 HRP PCR reactions were extracted and purified separately.

Concentrations:

1) 6.5 ng/ul 1.625 OD280/OD269
2) 5.0 ng/ul 2.0
3) 9.0 ng/ul 1.385
4) 5.5 ng/ul 2.2
5) too low
6) too low

1-4 were combined and measured at 6.5 ng/ul and 1.625 OD280/OD260. About 75 ul worth.

Digestion

Digests of gel extracted HRP with Eco and Pst to set up ligation into psb1c3 vector plasmid. Had to use all extracted product since concentration was so low. 50ul total volumes. 2x.

- 5ul 3.1 10x NEbuffer
- 0.50ul EcoRI
- 0.50ul PstI
- 35ul HRP
- 9.0ul H2O

Digested for 1 hour at 37 C followed by heat kill at 80 C for 15 mins. PstI cannot be heat killed at 65 C

Purification

Purified digested DNA and increased concentration by using the General DNA Cleanup from Enzymatic Reactions protocol with the Thermo Scientific Gel Extraction and DNA Cleanup Micro Kit.

1 Adjust the volume of the reaction mixture to 200 µL with Water, nuclease-free or TE buffer (not included).

2 Add 100 µL of Binding Buffer. Mix thoroughly by pipetting.

3 Add 300 µL of ethanol (96-100%) and mix by pipetting.

4 Transfer the mixture to the DNA Purification Micro Column preassembled with a collection tube. Centrifuge the column for 30-60 seconds at 14,000 × g. Discard the flow-through. Place the DNA Purification Micro Column back into the collection tube. Note. 1. If DNA fragment is ≥ 10 kb centrifuge the column for 2 minutes at 14,000 × g. 2. Close the bag with DNA Purification Micro Columns tightly after each use!

5 Add 700 µL of Wash Buffer (supplemented with ethanol, see p. 3) to the DNA Purification Micro Column and centrifuge for 30-60 seconds at 14,000 × g. Discard the flow-through and place the purification column back into the collection tube. Note. If DNA fragment is ≥ 10 kb centrifuge the column for 2 minutes at 14,000 × g.

6 Repeat step 5.

7 Centrifuge the empty DNA Purification Micro Column for an additional 1 minute at 14,000 × g to completely remove residual Wash Buffer. Note. This step is essential to avoid residual ethanol in the purified DNA solution. The presence of ethanol in the DNA sample may inhibit downstream enzymatic reactions.

8 Transfer the DNA Purification Micro Column into a clean 1.5 mL microcentrifuge tube (not included).

9 Add 10 µL of Elution Buffer (**added 20ul**) to the center of the DNA Purification Micro Column membrane. Centrifuge for 1 minute at 14,000 × g to elute DNA. Note. • If DNA fragment is ≥ 10 kb the elution volume should be increased to 15-20 µL to get optimal DNA yield. • Lower volume of Elution Buffer for DNA Micro Kit can be used (6-10 µL) in order to concentrate eluted DNA. Please notice that < 10 µL elution volume slightly decreases DNA yield. • Double the elution volume or perform two elution cycles when purifying larger amounts of DNA (for example > 5 µg).

10 Discard the purification column and store the purified DNA at -20°C.

Results

Concentrations of 22.5 ng/ul and 27.5 ng/ul with OD280/OD260 of 1.800 and 1.833, respectively. Combined samples -> 40ul of 25 ng/ul digested HRP.

Ligations

Ligated HRP into psb1c3. 2 20ul ligation reactions. 4 : 1 insert to vector molar ratio. One ligation with the newer NEB T4 Ligase and Buffer, the other with past expiration T4 Ligase and Buffer from Fermentas.

- NEB
    - 2ul 10x T4 DNA Ligase Buffer
    - 2ul psb1c3 (12.5 ng/ul..digested earlier with eco and pst)
    - 2ul HRP (25ng/ul)
    - 13ul nuclease free H2O
    - 1ul T4 ligase

- Fermentas
- 4ul 10x T4 Ligase Buffer
- 2ul psb1c3

- 2ul HRP
- 10ul nuclease free H20
- 2ul T4 Ligase

Both ligation reactions incubated at 16 C overnight (12 hours) followed by heat kill of 65 C for 10 mins. Then placed in -20 C.

June 26, 2015

Transformations

Transformed overnight ligations of HRP + psb1c3 into Thunderbolt GC10 Sigma electrocompetent cells with help from Mudassar. 1 transformation for each of the 2 ligation reactions plus 1 transformation as positive control using RFP + psb1c3 at 10 pico/ul provided by company.

- 33 ul competent cells + 2 ul Ligation A product. Produced an 'arc' during pulsing, tried again and showed no arc.
- 33 ul competent cells + 1 ul Ligation B product
- 33 ul competent cells + 1 ul positive control

Immediately added SOC recovery media after pulsing and placed in shaking incubator for 1 hour. Mudassar then plated on agar with chloramphenicol plates. Incubated at 37 C overnight.

PCR using high fidelity taq polymerase/master mix

As PCR using normal Qiagen Taq master mix on CBDA synthase was unsuccessful, PCR was attempted using the high fidelity version. A gradient was used to seek optimal annealing temperature for IDT Primers on constructs using high fidelity master mix. 8 reactions containing CBDA synthase plus 3 samples containing HRP were done with each CBDAS at a different annealing temperature and HRP samples spaced about 5 C apart.

Running out of old construct of CBDA synthase. Began with only 20ul of 10ng/ul. Need to dilute more before PCR'ing...down to 1ng/ul?

CBDA Synthase-

- 25ul 2x Mastermix High Fidelity
- 2.5ul 10uM IDT Primer R
- 2.5ul 10uM IDT Primer F
- 0.5ul CBDA synthase (sample A was 1ul, all others 0.5ul)
- 19.5ul dH2O

HRP-

- 25ul 2x Mastermix High Fidelity
- 2.5ul 10uM IDT Primer R
- 2.5ul 10uM IDT Primer F

- 1.0ul HRP
- 19ul dH2O

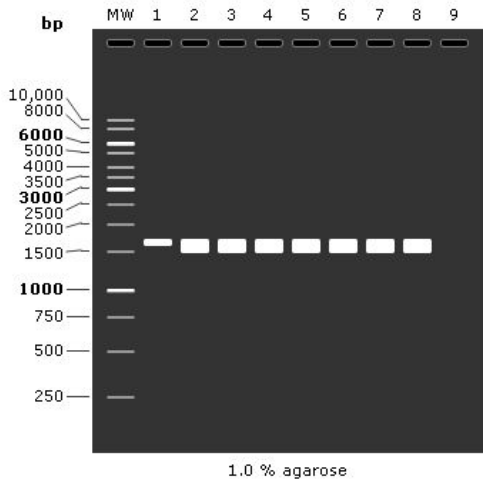
Gradient -

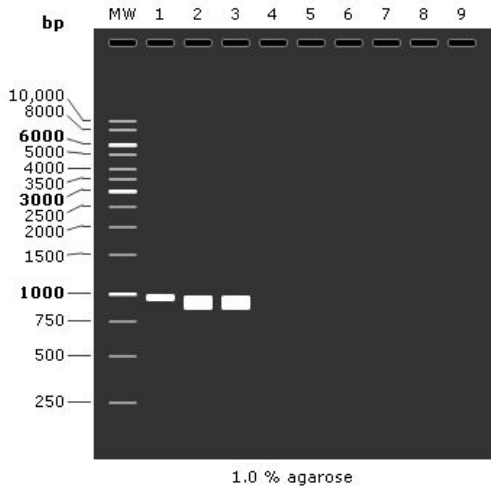
- 67 C - CBDAS sample A / HRP sample X
- 66 C - B
- 64.3 C - C
- 61.8 C - D / Y
- 58.5 C - E
- 56.2 C - F / Z
- 54.3 C - G
- 53 C - H

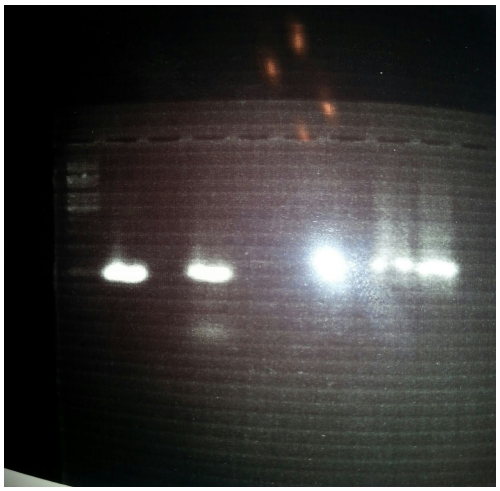
Cycle -

- 98 C - 30 s
- 98 C - 10 s -> 43 C - 30 s -> 72 C - 60 s ----- 5x
- 98 C - 10 s -> gradient C - 30 s -> 72 C - 60 s -----35x
- 72 C - 120 s
- 4 C - hold

1% Agarose Gel







Gel showed expected bands for CBDAS for A, B, and C. D through H had multiple amplifications. HRP samples X and Z had successful amplifications. Y showed no band.

June 30, 2015

Constructs-

Constructs with RFC 25 were mistakenly made with an extra nucleotide after EcoRI cut site. Different forward primer would have to be made to include the base. This extra base will be removed while ligating into psb1c3 by cutting at xba/pst instead of eco/pst. Also, standardized reverse primer was ordered incorrectly, need to reorder. Should be working with 3 primers to amplify mambalgin, alpha secretion, HRP, CBDAS constructs. (RFC 10 forward primer, RFC 25 forward primer, RFC 10/25 reverse primer).

Received notice from IDT that CBDAS is unable to be made due to unknown reasons. Will try to remake, maybe split into pieces.

July 6, 2015

New primers (RFC 10/25 reverse, RFC 25 forward) and constructs (alpha secretion, mambalgin) arrived.

PCR

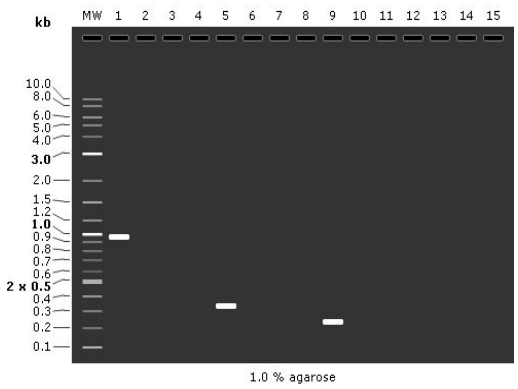
Test PCR with new primers on AS, mambalgin, and HRP constructs. 4x each construct. Temperature gradient 55.4 C, 52.6 C, 50.6 C, 47.8 C. 1-4 AS, 5-8 mambalgin, 9-12 HRP.

20ul final volumes.

- 10ul Qiagen taq master mix
- 1 ul IDT G-Block F or 10 primer
- 1 ul IDT G-Block 10/25 reverse primer
- 1 ul 1 ng/ul template
- 7 ul nuclease free h2o

94 C (3 mins) - 94 C (1 min) - Gradient (1 min) - 72 C (1 min) - 72 C (10 mins) - 4 C

1% Agarose gel, gelred, TAE, 2 log ladder



pGAP Vector

Innoculated overnight liquid cultures with glycerol stocks of possible pGAP vectors for expression in pichia pastoris. pGAPza A with linker, pGAPza B with linker, and pGAPza C with linker. Basically pGAPza plasmid with replaced multiple cloning site. The 2014 GSU iGEM team made pGAPza A, B, C iGEM standardized with RFC 10 prefix/suffix in MCS.

Made in LS LB. 25 mg/ml zeocin. 5ml. Will grow up, miniprep, digest/sequence to be sure of contents. 18 different samples.

PCR

PCR of mambalgin, alpha secretion, and HRP for downstream ligation into pSB1C3.

6x each construct. 50ul final volumes. Promega/Quiagen 2x master mixes. 2.5ul for/rev primers, 2.5ul 1ng/ul templates.

94 C (3 mins) – 94 C (1min) – 51 C (1min) – 72 C (1min) – 72 (10 mins) step 2 – 4 35x cycles.

Ran gel with 10ul of product which confirmed correct amplification, then purified using PCR cleanup protocol from Thermoscientific DNA Cleanup Kit.

Concentrations – HRP – 60 ng/ul 1.500 260/280

- AS – 90 ng/ul 1.676 260/280
- Mambalgin – 95.0 ng/ul

Have about 110 ul of each purified construct. 260/280 not great, should be above 1.8. Will still digest then ligate.

July 7, 2015

Muddassar doing digestions of purified alpha secretion, mambalgin, HRP PCRs. Have to cut with xba/pst for AS and mambalgin to remove extra nucleotide after RFC 25 ecorI cut site. HRP will be cut with eco/pst. 100ul volumes. 2ug DNA per digest, FD enzymes and buffer, 1 hour digestion 37 C + 15 min heat kill 80 C.

Minipreps of overnight liquid cultures of pGAP vectors yielded no plasmids. Low concentration for all 18 samples. Need to redo. Muddassar did more overnight cultures.

Muddassar also cutting pSB1C3 + promoter stock for pSB backbone for ligations. Need xba/pst cut backbone, not just eco/pst which is provided by iGEM.

July 8, 2015

Laura miniprepped overnight cultures, low concentrations but good enough. 4 sent for sequencing.

Constructs

Created mambalgin construct for expression in ecoli. Used same mambalgin sequence as other construct and added IPTG inducible promoter, ecoli RBS, and myc, 6x his epitope tags.

Also made new CBDA Synthase construct for expression in tobacco. Codon optimized using codon optimization tool on IDTdna.com. Then had to increase GC content from 38% to over 45% by changing codons manually. Low GC content is possibly the reason for the unsuccessful synthesis of previous CBDAS. Also, changed nucleotides to disable pstI site within coding sequence while keeping same amino acid.

Miniprep

Did some minipreps of Zahra's overnight cultures. 18 plus hours incubation. Testing qiagen miniprep kit. Followed Plasmid DNA Purification using Qiaprep columns protocol. Did different amounts of culture 0.75 ml to 4.00 ml. Eluted 30ul. Got too low concentration for nanodrop spec to read with 50 lid factor. Cultures being left to incubate too long? Bad buffers in qiagen kit?

July 9, 2015

Cancelled CBDAS construct as there was an eco site within coding sequence. Not iGEM compatible.

July 10, 2015

Constructs

EcoRI site removed from CBDAS construct by changing nucleotide but keeping same amino acid. Cancelled mambalgin construct. Did away with standardized primers for all constructs. Added different additional nucleotides to 'mambalgin for ecoli' and CBDAS RFC 10 prefix and suffix. Made forward and reverse primers for each construct. Checked possible hairpins and homo/heterodimeric structures using IDT 'Oligoanalyzer' tool. Change in gibbs free energy better than -3 cutoff. CBDAS primers made for Phusion polymerase – used Tm calculator provided on Phusion site to create annealing temperature below 72 C.

pGAP Vector

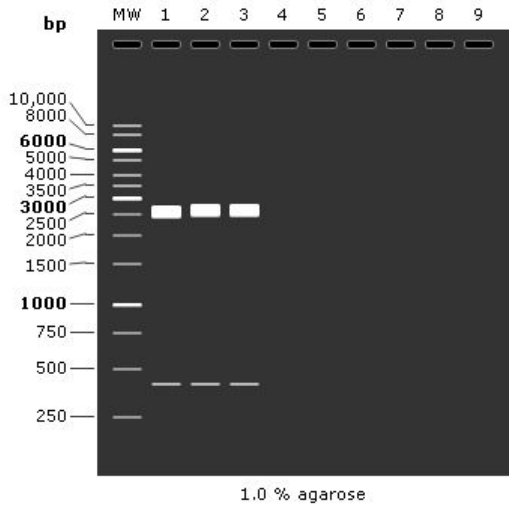
Did confirmation digests of miniprepped possible pGAP vectors. Gel showed no trace of DNA. Ladders visible. Bad cultures or miniprep kit?

Reza from last year's team gave me miniprepped samples of pGAP vectors. pGAPzaA + linker, pGAPzaB + linker, pGAPzaC + linker. Did confirmation digests, looked good.

- 500ng DNA
- 1.5 ul BamHI FD
- 1.5 ul PstI FD
- 2.0 ul FD Buffer
- dH2O

Digest 37 C – 20 mins

1% Agarose Gel.



Resulting gel had bands matching predicted.

July 15, 2015

Mamba for Ecoli construct and primers arrived. Tran and Zahra suspended and ran test PCR.

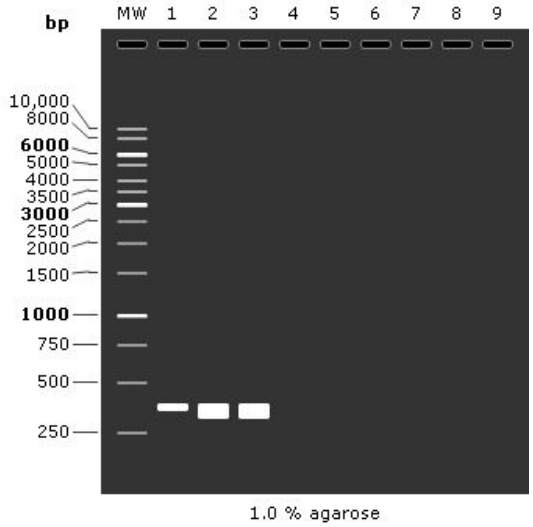
PCR

20ul final volumes. High fidelity polymerase master mix. Found annealing temperature using NEB Tm calculator.

- 1 ul 1ng/ul mambalgin for ecoli template
- 1 ul mambalgin F primer
- 1 ul mambalgin R primer
- 10 ul 2x master mix
- 7 ul dh2o

Followed rest of NEB high fidelity protocol. 67 C annealing temperature. 35x cycles.

1% gel was then ran.



Resulting gel had bands matching predicted.

Colony PCRs

Muddassar ligated alpha secretion and mambalgin for yeast into pSB1C3. Cut at xba/pst for constructs and backbone. Transformed into ecoli. Also transformed Reza's samples of pGAPza A + linker and pGAP za C + linker. Plated and grew overnight. Now colony PCR.

1-4 – Mambalgin for Yeast

5-8 – Alpha Secretion

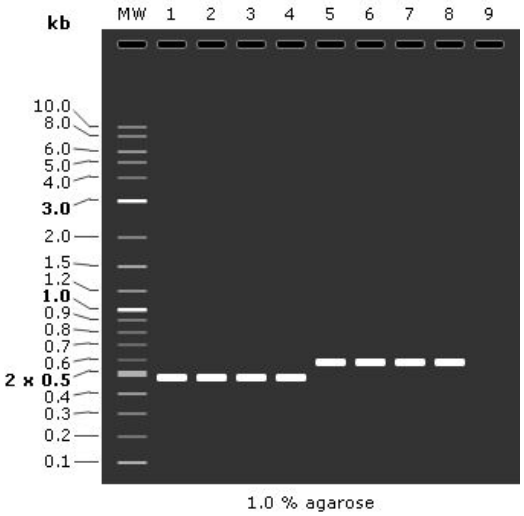
9-12 – pGAPza A + linker

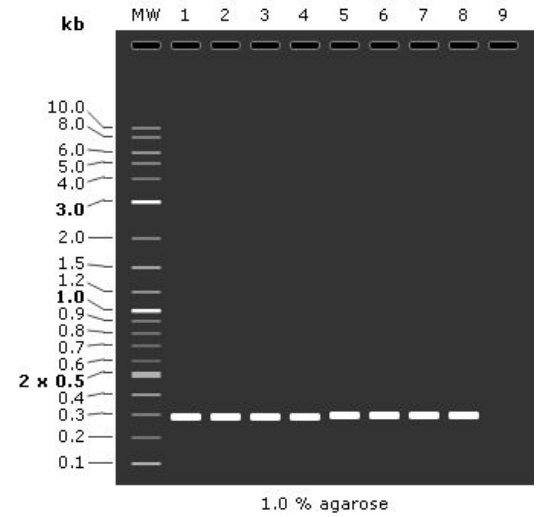
13-16 – pGAPza C + linker

Vf and Vr primers used for constructs in pSB, pPIC F and pPIC R used for pGAP vectors.

Liquid cultures also made in preparation for overnight incubation. Constructs in pSB in LB with 35 ug/ml chlorophenicol. pGAP vectors in LSLB with 25 ug/ml zeocin antibiotic. 5ml each.

Colony picked with pipette tip, dipped in PCR solutions, then left in overnight liquid cultures.





1-4, 5-6, 13, 15, 16 looked good. Miniprep those. Just need pGAPza C + linker for vector.

July 16, 2015

Minipreps

All liquid cultures were cloudy after 16 hour incubation in 37 C 250 rpm shaker. Glycerol stocks made – 0.500 ml culture plus 0.500 50% glycerol. Put in -80 C.

Followed miniprep protocol in qiagen miniprep kit. Eluted with 30 ul EB.

Concentrations – combined -

- Mambalgin for yeast in pSB1C3 – 92.5 ng/ul 1.850 260/280
- Alpha secretion in pSB1C3 – 75 ng/ul 1.875 260/280
- pGAPza C + linker – 47.5 ng/ul 2.111 260/280

Confirmation digests – NotI enzyme used with constructs in pSB1C3. BamHI and XbaI for pGAP vector.

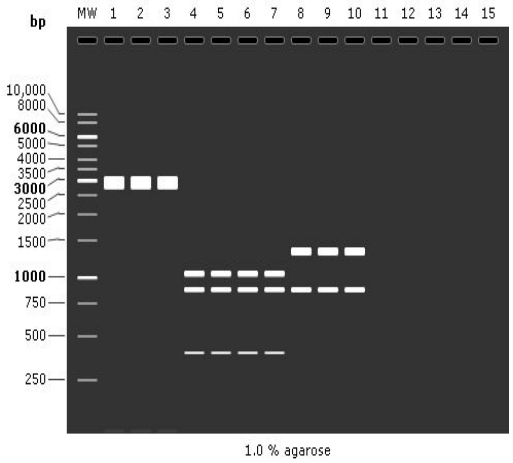
Repeated twice. Both resulted in very faint bands at top of ladder. Not as expected. Used 500 ng DNA. Tried uncut plasmids, very faint expected band. Tried PCR of minipreps – showed amplification of correct lengths. Plasmids there just in much lower concentrations. Miniprep products not pure? A230 high? Problem with buffers?

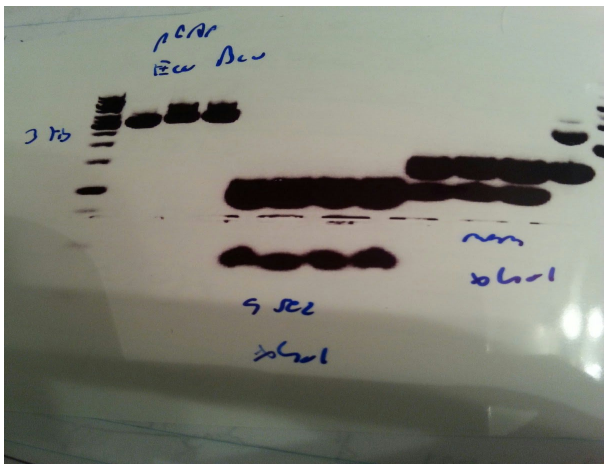
July 18, 2015

Tried confirmation digests of previous minipreps. Gel showed no DNA.

June 20, 2015

Muddassar had good confirmation digests of alpha secretion in pSB1C3, mambalgin for yeast in pSB1C3, and pGAP vector from his own cultures/minipreps. Gel showed expected bands.





Alpha secretion and mambalgin for yeast look like they've been ligated in pSB1C3. Send for sequencing.

Sequencing

pGAPza C + L, alpha secretion in pSB1C3, and mambalgin for yeast in pSB1C3 minipreps sent to ACGT for sequencing. 20ng/ul 10 ul DNA (from ~200ng/ul) minipreps. 2ul 5uM primers. 12 ul total in one eppendorf. pPIC F primer for pGAP, Vf primers for pSB

July 21, 2015

Digestions

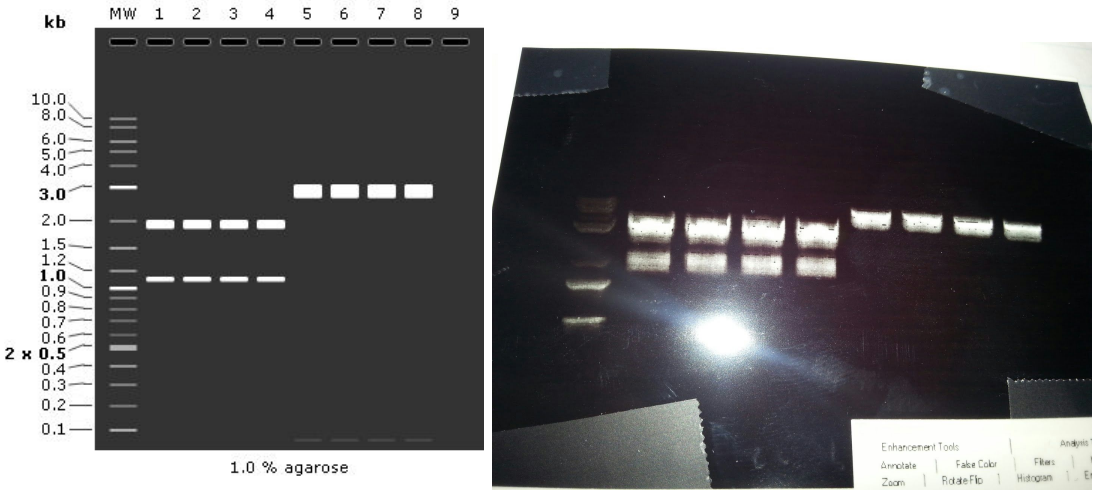
Need to create backbones for ligations into pSB1C3 and pGAPza C + linker. Cutting from stock of RFP in pSB1C3 (332 ng/ul) and pGAPza C + linker (155 ng/ul) plasmid with EcoRI and PstI enzymes for both.

- 2 ug DNA
- 5 ul FD Green Buffer
- 2.5 ul ecoRI
- 2.5 ul pstI
- nuclease free H2O

50 ul final volumes. 4 digestions each plasmid.

37 C - 30 mins

Digestion products ran on 1% agarose gel TAE 60 V, 2 hours.



Gel Extracted using Thermoscientific DNA cleanup kit, gel extraction protocol. Eluted 15 ul EB. Spectrometry showed ~30ng/ul concentration but high A230 OD because of agarose contamination. Worthless samples.

July 23, 2015

Muddassar was able to ligate HRP into pSB1C3. Colony PCRs and confirmation digests of minipreps look good. Ready for sequencing. Sent to GENEWIZ.com - followed recommended template/primer concentrations and mailed.

July 27, 2015

Mambalgin for ecoli construct arrived. Needs to be ligated into pSB1C3. PCR - Purify - Digest - Purify - Ligate

PCR and PCR purification done by Zahra and Tran.

Digests

PCR product of mamba for ecoli

- 20 ul DNA (100 ng/ul)
- 2 ul EcoRI-HF
- 2 ul PstI-HF
- 5 ul Cutsmart Buffer
- 21 ul nuclease free water

Incubation - 37 C - 1 hour, 80 C - 5 mins heat inactivation

RFP in pSB1C3 to create backbone for ligations.

- 4 ul DNA (500 ng/ul)
- 2 ul EcoRI

- 2 ul PstI
- 5 ul 3.1 NE Buffer
- 37 ul dh2o

Incubation - 37 C - 3 hours

Cleanups

Mambalgin for ecoli digest product purified using enzymatic protocol in Thermoscientific General DNA Cleanup Kit. Eluted 20 ul EB

Concentration - 120 ng/ul 1.920 A260/A280 ~70 ul combined.

pSB1C3 backbone gel extracted using gel extraction protocol in Thermoscientific General DNA Cleanup Kit.

Concentration - Too low

July 30, 2015

Sequencing confirms alpha secretion in pSB1C3 and mambalgin for yeast in pSB1C3. Ready to be submitted as parts to iGEM. Sequencing of 'pGAPza C + linker' showed pGAPza B plasmid with no iGEM modified MCS. Previous confirmation digests would also match that plasmid. Will modify constructs to insert into pGAPza B since there is 1.5ml of it. pGAPza A, B, C with linkers might not exist.

Sent 20 more samples to be sequenced by Genewiz. Including HRP in pSB1C3, more mamba for yeast in pSB1C3, RFP in pSB1C3, old 2014 mamba construct.

July 31, 2015

Muddassar ligated mambalgin for ecoli construct into pSB1C3. PCR and confirmation restriction digests look good.

August 3, 2015

Constructs

Mambalgin for yeast, HRP, CBDA Synthase all need to be inserted into pGAPza B vector. Designed primers to modify those constructs for in-frame ligation with alpha secretion and epitopes in pGAPza B. They remove RFC 10 prefix and suffix and replace them with an EcoRI site before the coding sequence, and an XbaI site after the coding sequence. These sites were on the 5' tail of the primers – Eco in forward primer, Xba on reverse primer. Used Snapgene to ensure proper framing. Had to add nucleotides after Eco site and before xba site for framing, as well as at the end of the 5' tails for optimal restriction enzyme cutting.

Mambalgin for yeast and HRP primers designed for Qiagen Taq polymerase master mix – annealing temperature ~5 degrees lower than Tm. CBDAS primers designed for Q5 high fidelity polymerase – annealing temperature judged using NEB Tm calculator. Primers checked for hairpins/primer dimers using IDT oligoanalyzer tool. Ordered from IDT.

Ordered a CBDA Synthase construct in 2 pieces and a kit for Gibson Assembly. CBDAS construct in RFC 25/Frieburg Standard.

Checked insertion of CBDAS and HRP into pORE E2/E4 agrobacterium vectors to be used for expression in tobacco. Did this on Snapgene. Requires no modifications using primers.

August 5, 2015

Modifying primers for mambalgin for yeast, HRP, CBDA Synthase constructs arrived.

PCR

Mambalgin for yeast, HRP

- 10 ul Qiagen Taq master mix
- 1 ul Mamba EcoRI Primer F or HRP EcoRI Primer F
- 1 ul Mamba XbaI Primer R or HRP XbaI Primer R
- 1 ul 1ng/ul DNA
- 7 ul nuclease free H2O

- 94 C (3 mins) – 94 C (1 min) – 56 C (1 min) – 72 C (1 min) – 5x – 94 C (1 min) – 62 C (1 min) – 72 C (1 min) – 30x – 72 C (10 min) – 4 C

CBDA Synthase

- 5 ul Q5 HF Buffer
- 0.5 ul dNTPs
- 1.25 ul CBDAS EcoRI Primer F
- 1.25 ul CBDAS XbaI Primer R
- 1 ul 1ng/ul CBDAS construct
- 0.25 ul Q5 Polymerase

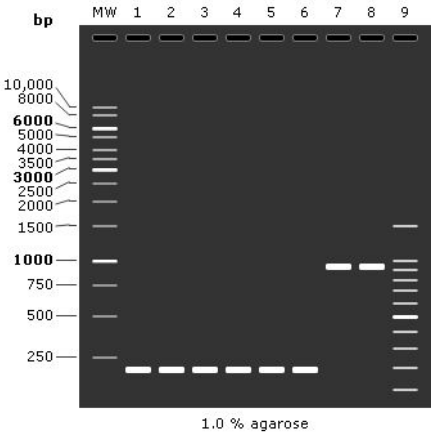
- 15.75 ul nuclease free H2O

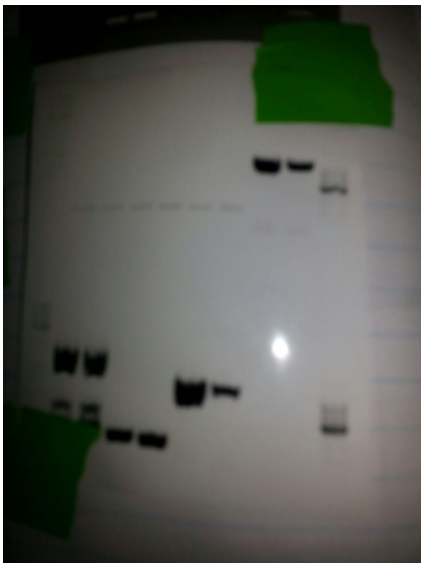
- 98 C (30 sec) – 98 C (10 sec) – 55 C (30 sec) – 72 C (60 sec) – 5x – 98 C (10 sec) - 70 C (30 sec) – 72 C (60 sec) – 72 C (120 sec) – 4 C

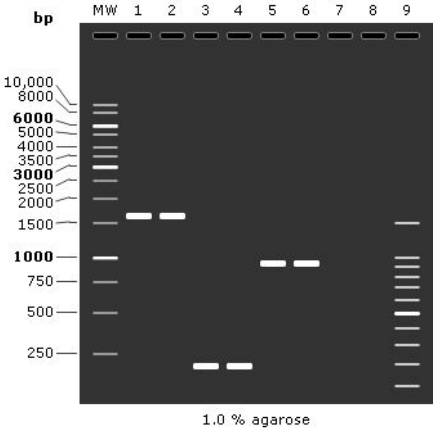
Also did some of mambalgin for yeast and HRP using Q5 polymerase.

Followed by 1% agarose gel. 2 ul of Mamba for yeast PCR reactions. If good – PCR cleanup of remaining products. Top row - Qiagen taq polymerase - 1-6 mambalgin for yeast PCR modified, 7-8 HRP PCR modified

Bottom row - Q5 polymerase - 1-2 CBDAS PCR modified, 3-4 mambalgin for yeast, 5-6 HRP







Modified mambalgin for yeast and HRP showed bands that matched expected. HRP had multiple amplifications, 1 strong band at correct length and 1 light smaller band. CBDAS showed strong amplifications at expected size and one smaller.

August 6, 2015

PCR

HRP with modifying primers for pGAPza B insertion. 50 ul volumes because want to PCR cleanup and digest. 6x.

- 25 ul Qiagen taq master mix
- 2.5 ul HRP EcoRI primer F
- 2.5 ul HRP XbaI primer R
- 1.0 ul 1ng/ul HRP construct
- 19.0 ul nuclease free H2O

Tran did PCR cleanup. Will do confirmation gel.

Digests

Need more pGAPza B and pSB1C3 backbones. Cutting with EcoRI and XbaI for pGAPza B, EcoRI and PstI for pSB1C3. Will do enzymatic cleanup of pGAP, gel extraction of pSB since coming from RFP + pSB1C3 stock. pGAP has very small insert being removed. 100 ul volumes. 6000 ng DNA

pGAPza B – 6x

- 35 ul DNA
- 2 ul EcoRI-HF
- 2 ul XbaI
- 10 ul 10x Cutsmart buffer
- 51 ul dH2O

37 C overnight incubation.

pSB1C3 + RFP – took from both a miniprep and maxiprep – 6x

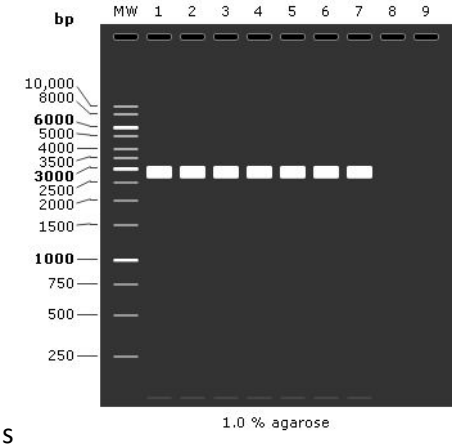
- 6000 ng DNA

- 2 ul EcoRI – HF
- 2 ul PstI – HF
- 10 ul 10x Cutsmart buffer
- dH2O

Gels

Did 1% agarose 50 ml TBE confirmation gel for pGAPza B digest (1-7) and HRP modifying PCR cleanup (8-13). Also 1% 100 ml TBE gel for extraction of pSB1C3 backbone.

Confirmation Gel - top row - 1-7 pGAPza B cut eco/xba. bottom row - all HRP PCR



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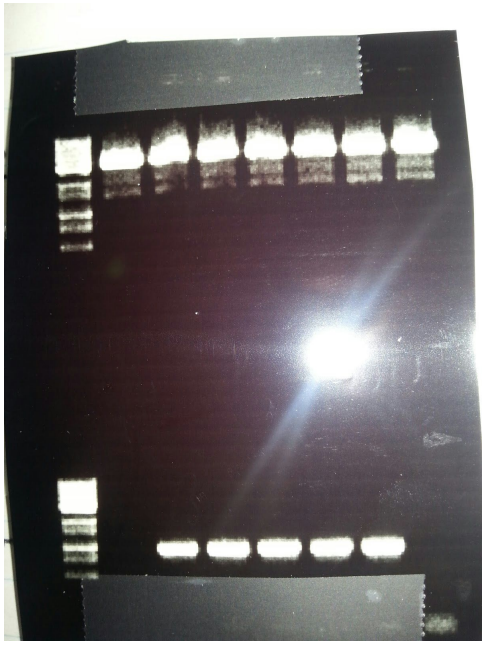
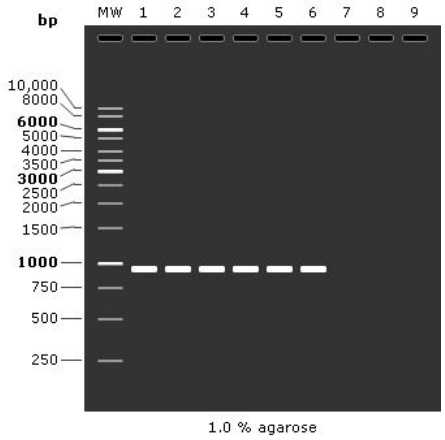
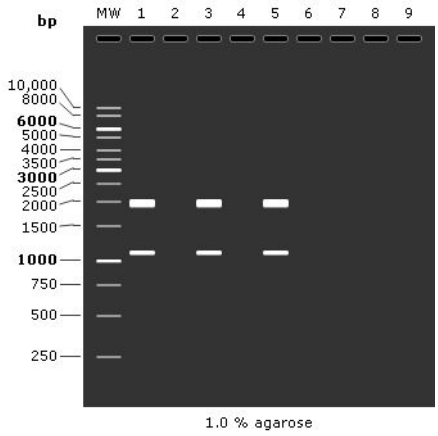
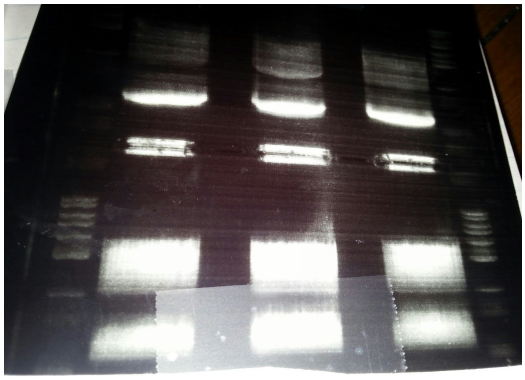




Top row shows possible star activity..multiple cuts. Strong band is at correct length, however. Bottom row shows no amplification in one PCR reaction - that sample thrown away.

Extraction Gel - top and bottom rows same predicted





Bottom row unusable. Top row shows less smearing and an overran gel. RFP band in bottom row wells. Will try to extract psb backbone in top row.

Cleanup

Did an enzymatic cleanup using the Thermoscientific General DNA Cleanup kit on the pGAPza B backbone. 15 ul elution using EB

Final concentration of 212 ng/ul, 1.809 A260/A280, 3.036 A260/A230 for ~90 ul purified pGAPza B backbone.

Did a gel extraction of the pSB1C3 backbone using the gel extraction protocol in the Thermoscientific General DNA Cleanup kit. 15 ul elution using EB

Concentrations showed some agarose contamination. A260/A230 of less than 1.0 for most samples. Sample B was ok – 115 ng/ul, 1.769 A260/A280, 1.769 A260/A230. Will use for ligations.

Ligations

CBDA Synthase into pSB1C3 - ~1.5/1 insert/vector molar ratio

- 1.0 ul CBDAS (eco/pst cut) 250 ng/ul
- 2.0 ul pSB1C3 purified (eco/pst cut) 115 ng/ul
- 1.0 ul T4 Ligase
- 2.0 ul 10x T4 Ligase buffer
- 14.0 ul dH2O

16 C (overnight) – 25 C (15 min) – 65 C (15 min)

Mambalgin for yeast in pGAPza B - ~5/1 insert/vector molar ratio

- 3 ul mambalgin for yeast (eco/xba) 135 ng/ul
- 4 ul pGAPza B backbone (eco/xba) 200 ng/ul
- 1 ul T4 Ligase
- 2 ul T4 Ligase buffer
- 10 ul dH2O

16 C (overnight) – 25 C (15 min) – 65 C (15 min)

August 7, 2015

Tranformations

CBDAS in pSB1C3 into ecoli, mambalgin for yeast in pGAPza B into ecoli – NEB 5 alpha chemically competent cells (C2987I). Followed protocol given by company. 5 ul ligation product in each transformation. Also did 10 pg RFP + pSB1C3 testing efficiency.

Plated with 50, 100, and 200 ul SOC + cells on zeocin + LSLB plates for mambalgin for yeast in pGAPza B. 50, 100, 200 ul SOC + cells on chloramphenicol + LB plates for CBDAS in pSB1C3. SOC diluted 1/10 before plating. Grew 37 C overnight.

Cleanup

Muddassar did enzymatic cleanup of HRP modified for pGAPza B after digesting with EcoRI/XbaI. Thermoscientific General DNA Cleaup kit. Actually used PCR cleanup protocol. Combined 2 digestion products into one before starting.

Concentrations - 1 – 153 ng/ul, 1.79 A260/A280, 2.54 A260/A230

2 – 130 ng/ul, 1.86, 3.47

3 – 210 ng/ul, 1.79, 2.47 – looked best

Ligation

Modified HRP into pGAPza B.

- 6 ul modified HRP (eco/xba cut) 200ng/ul
- 3 ul pGAPza B (eco/xba cut) 200 ng/ul
- 1 ul T4 Ligase
- 2 ul T4 Ligase buffer
- 8 ul dH2O

16 C (overnight) – 25 C (15 mins , lid temp 45 C) – 65 C (15 min, lid temp 95 C)

Overnight Liquid Cultures

Creating stocks of sequence – verified iGEM parts. Will miniprep.

HRP in pSB1C3 (4x, sample number 13), Alpha Secretion in pSB1C3 (4x, MH 6 sample), Mambalgin for yeast in pSB1C3 (4x, MH 12 sample) – from glycerol stocks.

10 ml LB 60 ug/ml chloramphenicol for selection. 37 C 250 rpm

August 8, 2015

Muddassar did transformation of HRP in pGAPza B into ecoli – NEB 5 alpha chemically competent

Plasmid Purification

Liquid cultures for alpha secretion in pSB, HRP in pSB, mambalgin for yeast in pSB were all cloudy. Did minipreps for each. All 10ml cells into 1 column.

Also did minipreps for Muddassar's mambalgin for ecoli in pSB liquid cultures. Just 2 looked good after colony PCR confirmation.

Followed miniprep protocol from Qiagen miniprep kit. Eluted 50 ul with EB.

Concentrations-

Mamba for ecoli - #7 – 143 ng/ul, 1.966, 1.390

- #19 – 215 ng/ul, 1.870, 1.790

Rest of constructs in pSB1C3 had consistent concentrations. Combined concentrations (~190 ul each) –

HRP in pSB1C3 – 158 ng/ul, 1.800, 1.703

Alpha secretion in pSB1C3 – 140 ng/ul, 1.867, 2.240

Mambalgin for yeast in pSB1C3 – 158 ng/ul, 1.854, 2.333

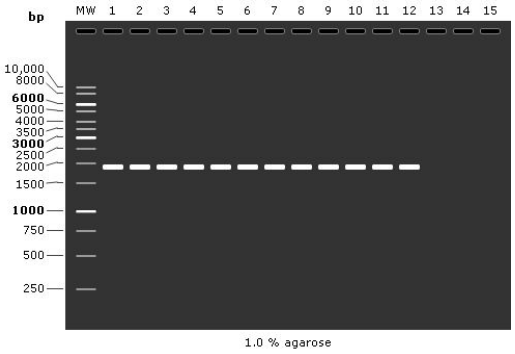
PCR

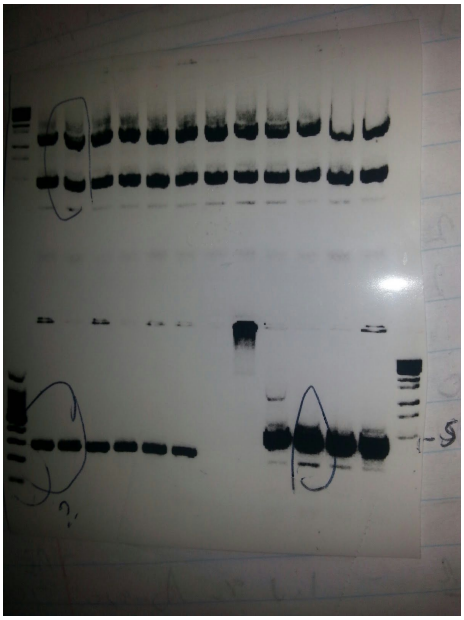
Colony PCR of plates of CBDAS in pSB1C3, mambalgin for yeast in pGAPza B. CBDAS in pSB – Vr/Vf primer with Q5 polymerase. Followed recommended cycle 35X with 66 C annealing. Mambalgin for yeast in pGAP – pPIC F and pPIC R with Qiagen Taq master mix. Followed recommended cycle 35x with 52 C annealing.

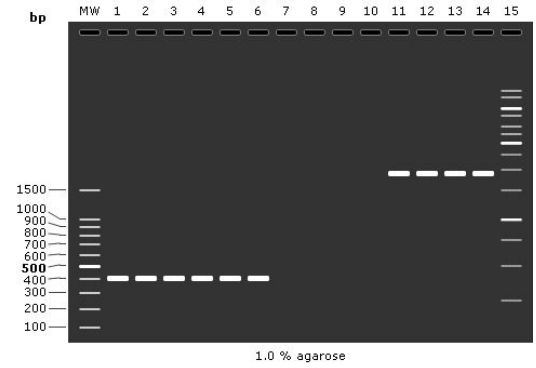
PCR of CBDAS construct using modifying primers for pGAPza B insertion. 50 ul volumes, 8x. Will gel extract since expecting multiple amplifications. Q5 polymerase.

Gels

Confirmation colony PCRs of CBDAS in pSB1C3 and mambalgin for yeast in pGAPza B. Top row- CBDAS in pSB1C3 PCR VF VR primers. Bottom row - 1-6 mambalgin for yeast in pGAPza B PCR pPIC F R primers. 9-12 - more CBDAS PCR

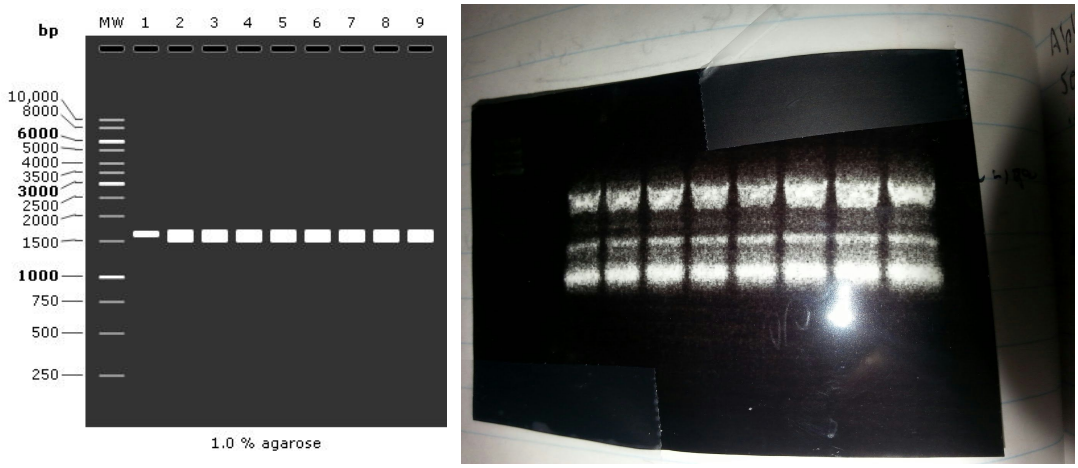






None of the colony PCRs match expected. Band size of ~ 300 bp indicates closed pGAPza B vector with no insert. Redo ligations of CBDAS in pSB1C3 and mambalgin for yeast in pGAPza B.

Gel for extraction of modified CBDAS for pGAPza B



Has multiple amplifications, one of which looks like the expected size of CBDAS insert. Can gel extract.

Cleanup

Gel extraction of modified CBDAS using Qiagen gel extraction kit with columns with purple caps. Followed protocol. Eluted 30 ul EB. Combined 1-4 and 5-8 bands before addition of QV. Concentrations measured with nanodrop spectrophotometer show a great amount of agarose contamination for 1 sample. Will not use. Other sample – 55 ng/ul, 2.200 A260/A280, 0.957 A260/A230. Will digest.

August 10, 2015

Digestion

Modified CBDAS PCR product, purified. Digest for ligation into pGAPza B. EcoRI/XbaI.

- 30 ul CBDAS PCR cleaned up (50ng/ul)
- 1.5 ul EcoRI – HF
- 1.5 ul XbaI
- 5.0 ul 10x Cutsmart buffer
- 12 ul dH2O

37 C – 1 hour incubation

Cleanup

Muddassar did cleanup using PCR purification protocol in Thermoscientific General DNA Cleanup kit. 20 ul elution with EB. Concentration – 185 ng/ul, 1.850, 1.762. Ready to be ligated into pGAPza B

Ligations

PCR modified mambalgin for yeast in pGAPza B, PCR modified HRP in pGAPza B, modified CBDAS in pGAPza B, unmodified CBDAS in pSB1C3. All ~5 to 1 insert to vector molar ratio. 20 ul final volume, 1 ul T4 ligase, 2 ul 10x T4 ligase buffer, dH2O.

16 C (overnight) – 25 C (15 mins) – 65 C (15 mins)

August 11, 2015

Transformations

Muddassar transformed overnight ligations into NEB 5 alpha chemically competent ecoli cells. Followed recommended protocol. Plated 50 ul SOC + cells onto 50 and 100 ug/ml zeocin+LSLB or chloramphenicol+LB plates. Grown overnight 37 C.

PCR

More colony PCRs of plates with mambalgin for yeast in pGAPza B. pPIC F, pPIC R primers. Qiagen taq master mix. Followed by 1% agarose gel, 50 ml TBE, 150 V, 40 mins.

Results showed same ~300 bp bands from previous colony PCR's. 4 liquid cultures containing growth from colonies were kept and miniprepped using qiagen miniprep kit. Concentrations were too low to be measured by nanodrop spectrophotometer. Thrown out.

August 12, 2015

PCR

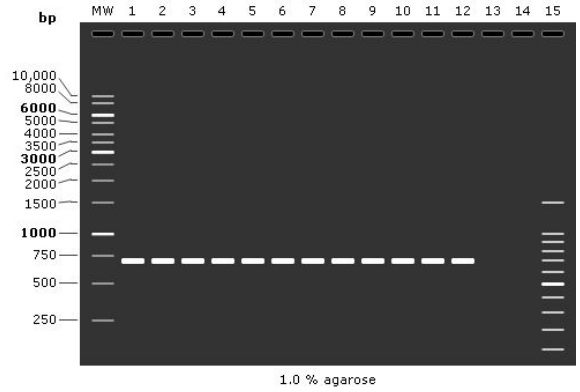
Colony PCRs of tranformations. Plates show growth. Muddassar doing HRP in pGAPza B and mambalgin for yeast in pGAPza B. I did CBDAS in pSB1C3 and CBDAS in pGAPza B colonies. Used new primers within CBDAS coding sequence so can work with cheaper qiagen taq master mix instead of Q5.

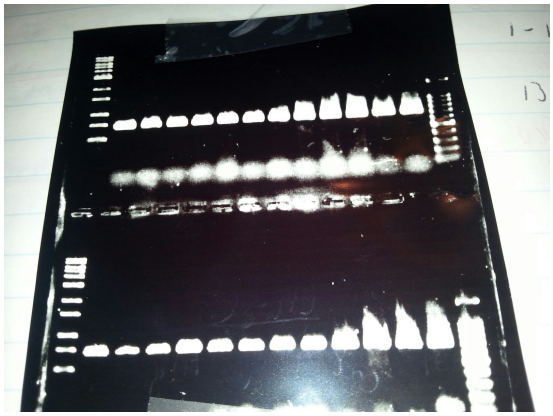
- 10 ul Qiagen taq master mix
- 1 ul CBDAS col pcr F
- 1 ul CBDAS col pcr R
- 8 ul dH2O

Picked colonies on plates with pipette tip, dipped in premade PCR mix, then dropped into LSLB with zeocin.

- 94 C (3 mins) – 94 C (45s) – 55 C (45s) – 72 C (45s) – 72 C (3 mins) – 4 C

Ran on 1% agarose gel 50 ml TBE. 12 each of CBDAS in pSB1C3 and CBDAS in pGAPza B. One positive control of known CBDAS construct. Top row - CBDAS in pSB1C3 PCR col pcr primers F R. bottom row - CBDAS in pGAPza B PCR col pcr primers F R. Same expected length for all.





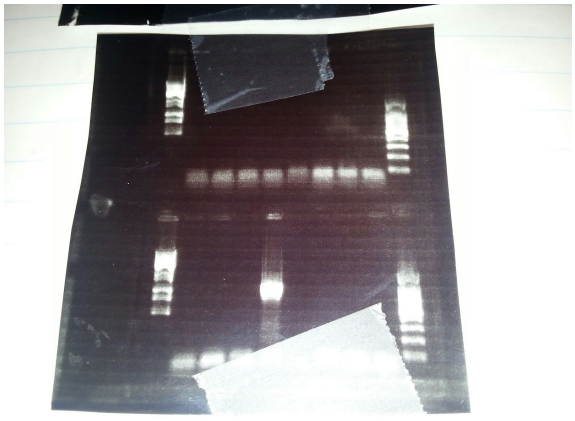
Results show all colonies with correct band size, matching positive control (last sample on gel). Unusual that every single colony had ligated plasmid. False positives? Try again with primer in vector backbone and another in coding sequence.

Retry – Same colonies picked but using pPIC R primer with CBDAS col pcr F primer. PCR ran overnight.

Muddassar's colony PCR's showed possible ligated plasmids in a few of the colonies. About half looked ok, the other half had wrong bands. The ones that looked good were put I liquid cultures for overnight growth. 2 of mamba for yeast in pGAPza B and 2 of HRP in pGAPza B. LSLB 10 ml, 75 ug/ml zeocin. 37 C 250 rpm. My CBDAS in pSB1C3 or pGAPza B liquid cultures were also grown overnight. 60ug/ml chloro+LB, 75 ug/ml zeocin+LSLB.

August 13, 2015

Gel of overnight PCR of CBDAS ligations showed no bands except for one colony. Will still try to miniprep some.



Minipreps

Plasmid purifying CBDAS ligations. 1-8 of CBDAS in pSB1C3, and 13, 14, 17, 18 of CBDAS in pGAPza B liquid cultures. Used Qiagen miniprep kit. Concentrations were ~100 ng/ul, ~1.800, ~2.000 for all. Confirmation digests yielded a gel with no bands/DNA.

Also miniprepped possible mamba for yeast in pGAPza B and HRP in pGAPza B liquid cultures. Concentrations – 4 – 392 ng/ul, 1.847, 19.6

6 – 268 ng/ul, 1.877

7 – 548 ng/ul, 1.856, 10

11 – 498 ng/ul, 1.793, 9.5

4 and 6 are mambalgin for yeast in pGAPza B

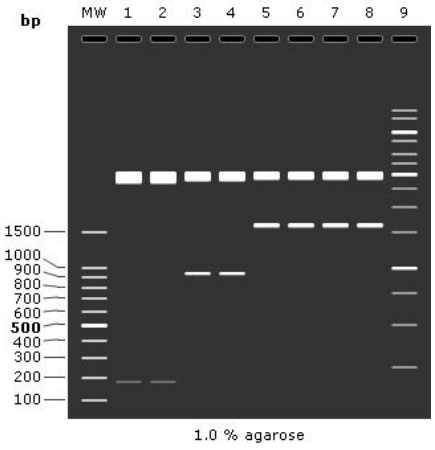
7 and 11 are HRP in pGAPza B

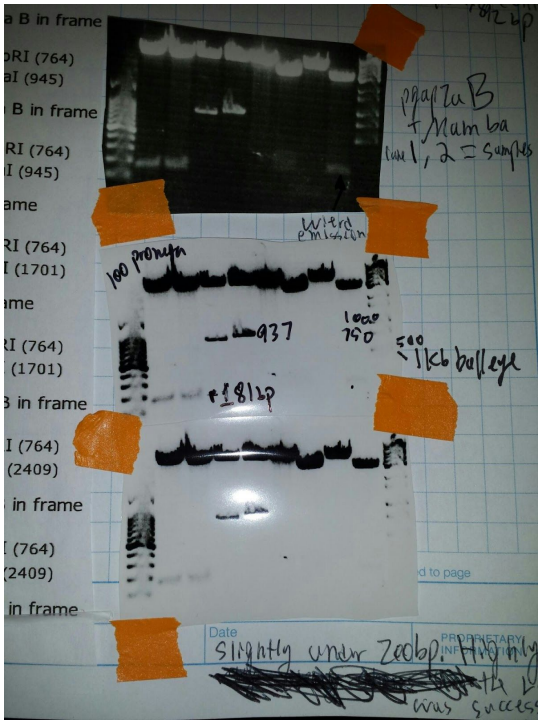
Digests

- 1-1.5 ug DNA
- 2 ul FD green buffer
- 1 ul EcoRI FD
- 1 ul XbaI FD
- dH2O

37 C – 45 mins

1% agarose gel, 80 ml TBE, 150 V 40 mins





1-2 mambalgin for yeast in pGAPza B, 3-4 HRP, 5-8 CBDAS. Digests look good for mambalgin for yeast in pGAPza B and HRP in pGAPza B. Need to be sent for sequencing. CBDAS digests did not yield expected bands. CBDAS is not ligated in pSB or pGAP. Also needs to be ligated into pORE E2/E4 when those vectors arrive. HRP also in pORE E2/E4. All other ligations completed.

August 24, 2015

Sequencing for mamba for yeast in pGAPza B and HRP in pGAPza B came back and look fine. Ready to be expressed.

August 26, 2015

Transformed mambalgin for ecoli in pSB1C3 into BL21 chemically competent cells. This is an expression strand. Followed protocol provided by NEB (C2530). Plated on chloro plates 60ug/ml and incubated overnight.

Constructs

Decided to design a construct for HRP expression in ecoli. HRP coding sequence was codon optimized, then removed iGEM non standard spe/eco/pst sites within coding sequence by changing nucleotides. Removed stop codon. Added inducible lac promoter, RBS, and epitopes..same ones as mambalgin for ecoli. RFC 10. Additional nucleotides same as mambalgin for ecoli - so same primers to amplify.

August 28, 2015

Plates of transformation of mambalgin for ecoli in pSB1C3 in BL21 expression ecoli had growth. Colony PCR showed expected bands. Ready to be expressed.

Starter culture - 10ml overnight LB cultures, 60ug/ml chloro. PCR confirmed colonies picked. Grown overnight 37 C 250 rpm.

August 31, 2015

Inoculated main expression cultures with 5 ml of cloudy starter cultures. 300 ml and 400 ml, 34ug/ml chloro - want good growth. Grown to ~0.56 OD 600 over a few hours. Mambalgin for ecoli has promoter inducible by IPTG. IPTG added 1mM to 300 ml culture and grown overnight at 20 C.

Realized incorrect antibiotic amount put into main cultures. 3.4 ug/ml instead of 34. Start over.

September 2, 2015

Mambalgin for ecoli starter cultures made previous day and incubated overnight. 6 ml LB + 34 ug/ml chloro. Cells from PCR confirmed colony.

Main cultures 400 ml and 400 ml LB + 34 ug/ml chloro inoculated with starter cultures and grown to 0.500 - 0.600 OD 600. One culture then IPTG induced 1mM between 3-4 hours at 37 C 250 RPM.

September 3, 2015

Cultures with hopefully expressed mambalgin in ecoli taken to Jessica's lab which has equipment necessary for protein extraction/characterization. Mudassar's expressed HRP in pichia also taken.

Cultures spun 8000 x g 20 mins. Pellet resuspended in lysis buffer with lysosome added, then used a french press for complete cell disruption. Product looked abnormal to Jessica. Product then centrifuged 5 mins.

Bradford Assay performed - showed 6 ug/ml protein present for mambalgin in ecoli.

SDS-PAGE

Done to determine protein sizes. 2 gels were made with same samples, with 1 going to be used for a coomassie stain and the other a western blot. Broad range 11-245 kda ladder used (p7712s).

Lane 1 - Ladder

Lane 2 - HRP 1

Lane 3 - HRP 2

Lane 4 - HRP 3

Lane 5 - Supernatant from first spin sample 2

Lane 6/7 - Supernatant from 2nd spin sample 1

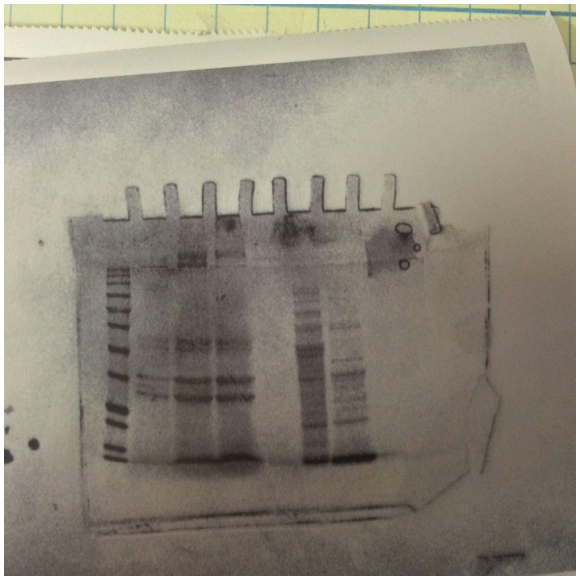
Lane 8/9 - Supernatant from 2nd spin sample 2

Ran for 30 mins 200 V.

Western Blot and Coomassie Stain

Western then done after transfer of SDS-PAGE gel to membrane using transfer machine 14 mins 24 V. Washed with 5% milk in dH2O solution 1 hour. Primary antibody added 1 hour. Poured off. Secondary also added 1 hour. Poured off. Antibodies for myc epitope. Results were no signal.

Coomassie done to other gel. Stained for 1 hour then destained overnight. Results showed possible HRP protein size in lanes 1-3 and Mambalgin protein size in the supernatant after lysis of sample 1 - lanes 5-6. HRP after alpha secretion peptide cleavage - ~38 kda. Mambalgin ~9 kda.



Process needs to be repeated, starting from mambalgin for ecoli and HRP for yeast starter cultures. Inexperienced in protein characterization procedures.